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(54) Title: METHOD OF MAKING MONOCLONAL ANTIBODIES USING POLYMORPHIC TRANSGENIC ANIMALS

(57) Abstract

The invention relates to a method for making monoclonal antibodies having pre–defined specificity of an epitope characteristic of or unique to a single form of a polymorphic protein. The method includes constructing a first transgenic animal to express a first form of a polymorphic protein encoded by a first allele of a gene encoding the protein; constructing a second transgenic animal to express a second form of the polymorphic protein encoded by a second allele of the gene encoding the protein; and immunizing the first transgenic animal with cells from the second transgenic animal expressing the second form of the polymorphic protein to induce an immune response in the first transgenic animal yielding an antibody specific for an epitope peculiar to the second form of the polymorphic protein. The invention further includes hybridoma cells secreting a monoclonal antibody specific for the second form of the protein. The invention is particularly advantageous in the context of making monoclonal antibodies and derivative reagents specifically identifying polymorphic blood group proteins, such as the Duffy gp–Fy protein.

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METHOD OF MAKING MONOCLONAL ANTIBODIES USING POLYMORPHIC TRANSGENIC ANIMALS

BACKGROUND OF THE INVENTION

The invention relates to methods of making antibodies. More specifically, the invention relates to the selective production of monoclonal antibodies having predefined specificity.

Almost a century ago, the discovery of ABO blood groups by Karl Landsteiner (1901) led to the development of routine blood grouping procedures in the practice of pretransfusion testing performed today. This testing depends on agglutination resulting from the interaction between the red blood cells (RBCs) and their cognate antibodies. Historically, human-source reagents have been used for blood group antigen typing. From time to time attempts have been made to use non-human reagents for blood grouping. Injection of human RBCs to animals, such as mice and rabbits, often results in production of a mixture of antibodies which require extensive absorption or purification for use to detect a single polymorphic determinant. However, with the advent of the ability to produce monoclonal antibodies (MAbs) having predefined specificities as described in the mid-seventies (Kohler et al. 1975), and in view of the legally restricted immunization of individuals with allogeneic RBCs, a new era began in the blood typing industry.

In producing these new monoclonal antibodies, after immunization of mice with appropriate antigens (e.g., RBCs), an antibody-secreting spleen cell is isolated and fused with an "immortal cell" (a myeloma cell line), to create a hybrid cell called a "hybridoma." The resulting hybridoma uniquely secretes the antibody of interest into the culture medium, but is also immortal, thereby capable of acting as a continuous source of the antibody.

The first murine MAb-based blood typing reagent, an anti-M, was licensed by the Food and Drug Administration in 1984 and since that date, MAb reagents for ABO typing have replaced those prepared from plasma from human donors. MAbs have the advantages of unlimited supply, batch-to-batch consistency, absence of contaminating antibody specificities, and minimal biohazard risk to blood typing staff.

While efforts have been concentrated on making MAbs using the mouse hybridoma system, new techniques keep on developing. In vitro infection of human B-lymphocytes with

Epstein-Barr virus (EBV) results in production of transformed, immunoglobulin-secreting lymphoblastoid cells which survive in tissue culture indefinitely and continue to secrete specific antibodies. EBV-transformed human lymphoblastoid cell lines can be fused with a mouse/human myeloma cell line to make MAbs. In this fashion, human anti-D, anti-C and anti-E have been made (Doyle et al. 1985; Crawford et al. 1983). However, the major limitation of this technique is the need for lymphocytes from recently immunized human donors. Thus, this is not an effective method for producing a broad range of blood grouping reagents.

Enriched RBC membrane proteins and synthetic peptides have also been used to immunize animals. Unfortunately, limited success has been obtained due to the inability of the animal to recognize blood group polymorphisms, or because the antigens are only expressed if the protein is embedded in the RBC membrane.

While monoclonal technology has advanced substantially and concerted efforts have been made in many institutions, it has not yet proven possible to make MAb reagents with certain specificities, notably anti-Fy^a and anti-Fy^b. In addition, as human source material, e.g., antibodies of sufficient potency, becomes harder and harder to obtain, it is inevitable that MAb reagents will be needed to replace polyclonal reagents. It is obvious that new approaches are needed if MAbs with specificities that are not currently available are to be made.

In view of the above considerations, it is clear that existing methods for making antibodies are limited. Moreover, it is evident that existing blood typing reagents based on antibodies are limited in both quality and quantity, necessitating new sources of such reagents.

Accordingly, it is one of the purposes of this invention to overcome the above limitations in the manufacture of antibodies, by providing a method which enables designed production of antibodies, particularly monoclonal antibodies to have particular pre-defined specificities. The availability of such designer antibodies thereby enables the manufacture and production of reagents and methods of detecting expression specific proteins which are presently either difficult or even impossible to identify by conventional methods. Therefore, it is another purpose of the invention to provide a method of making a wide range of MAbs capable of use for typing blood samples, investigating functions of proteins, and developing therapeutic reagents.

SUMMARY OF THE INVENTION

It has now been discovered that these and other objectives can be achieved by the present invention, which provides a method for making an antibody, comprising:

constructing a first transgenic animal to express a first form of an exogenous polymorphic protein encoded by a first allele of a gene encoding the protein;

constructing a second transgenic animal to express a second form of the polymorphic protein encoded by a second allele of the gene encoding the protein;

immunizing the first transgenic animal with cells from the second transgenic animal expressing the second form of the polymorphic protein to induce an immune response in the first transgenic animal yielding an antibody specific for an epitope characteristic of the second form of the polymorphic protein.

The method preferably further comprises isolating from the first transgenic animal a lymphoid cell capable of producing the antibody, as well as the cell isolated thereby. More preferably, the method further comprises fusing the isolated antibody-producing lymphoid cell with an immortal cell to provide an antibody-producing hybridoma cell.

The invention, therefore, provides a method of making a hybridoma cell which produces a monoclonal antibody having specificity for an epitope uniquely identifying or characteristic of a single form of a polymorphic protein. The invention further includes the hybridoma cell produced by the method, as well as the monoclonal antibody produced by the hybridoma cell.

The method of the invention can be employed in the context of an exogenous polymorphic protein which is expressed on a cell membrane. Preferably, the polymorphic protein is a blood group protein. More preferably, the blood group protein is gp-Fy protein. It is preferred that the polymorphic protein is a human polymorphic protein.

Preferably, the first transgenic animal and the second transgenic animal are both transformed from animals of one inbred strain. It is further preferred that the first and second transgenic animals are mice.

The invention further includes a hybridoma cell, preferably one that cannot routinely be made by conventional or standard methods known in the art. The hybridoma cell is produced by a method comprising:

constructing a first transgenic animal to express a first form of an exogenous polymorphic protein encoded by a first allele of a gene encoding the protein;

constructing a second transgenic animal to express a second form of the polymorphic protein encoded by a second allele of the gene encoding the protein;

immunizing the first transgenic animal with cells from the second transgenic animal expressing the second form of the polymorphic protein to induce an immune response in the first transgenic animal yielding an antibody specific for an epitope characteristic of the second form of the polymorphic protein;

isolating from the first transgenic animal a lymphoid cell capable of producing the antibody; and

fusing the antibody-producing lymphoid cell with an immortal cell to provide an antibody-producing hybridoma cell.

The hybridoma cell is preferably capable of producing an antibody specific for an epitope characteristic of a form of a polymorphic protein which is a blood group protein, more preferably being capable of producing an antibody specific for an epitope characteristic a form of gp-Fy protein.

The invention also includes an antibody specific for an epitope characteristic of one form of a polymorphic protein, produced by a method comprising:

constructing a first transgenic animal to express a first form of an exogenous polymorphic protein encoded by a first allele of a gene encoding the protein;

constructing a second transgenic animal to express a second form of the polymorphic protein encoded by a second allele of the gene encoding the protein;

immunizing the first transgenic animal with cells from the second transgenic animal expressing the second form of the polymorphic protein to induce an immune response in the first transgenic animal yielding an antibody specific for an epitope characteristic of the second form of the polymorphic protein; and

isolating the antibody.

The method preferably further comprises:

isolating from the first transgenic animal a lymphoid cell capable of producing the antibody; and

fusing the antibody-producing lymphoid cell with an immortal cell to provide a hybridoma cell which produces the antibody.

The antibody is preferably specific for a polymorphic protein which is a blood group protein, more preferably wherein the blood group protein is gp-Fy protein and the monoclonal antibody is specific for an epitope characteristic of one form of gp-Fy protein.

The invention also includes a method for making an antibody, comprising:

constructing a transgenic animal, preferably a transgenic mouse, to express a first form of an exogenous polymorphic protein encoded by a first allele of a gene encoding the protein;

immunizing the transgenic animal with a peptide comprising an epitope characteristic of a second form of the polymorphic protein to induce an immune response in the transgenic animal yielding an antibody specific for the epitope; and

isolating the antibody.

Preferably the method further comprises isolating from the transgenic animal a lymphoid cell capable of producing the antibody. Still more preferably the method further comprises fusing the isolated antibody-producing lymphoid cell with an immortal cell to provide an antibody-producing hybridoma cell.

Preferably, the polymorphic protein is expressed on a cell membrane, e.g., a blood group protein, such as gp-Fy protein. Preferably, the polymorphic protein is a human polymorphic protein.

These and other advantages of the present invention will be appreciated from the detailed description and examples which are set forth herein. The detailed description and examples enhance the understanding of the invention, but are not intended to limit the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the invention have been chosen for purposes of illustration and description, but are not intended in any way to restrict the scope of the invention. The preferred embodiments of certain aspects of the invention are shown in the accompanying drawing, wherein:

Figure 1 is a diagram of the proposed topography of the Duffy glycoprotein within the red cell membrane.

Figure 2 is a schematic illustration of a method of constructing transgenic mice expressing *FY*B*.

Figures 3A and 3B together constitute a schematic illustration of the Duffy genomic DNA sequence (*FY*B*) used to produce transgenic mice.

Figure 4 is a digitized image of PCR amplification products derived from Duffy genomic DNA sequence showing integration into the genome of transgenic mice.

Figure 5 is a schematic illustration of a method of producing monoclonal antibodies by the transgenic animal/hybridoma technique of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to a method of making antibodies having pre-defined specificity for one form of a polymorphic protein. The invention permits the manufacture of monoclonal antibodies (MAbs) with pre-defined specificities, including MAbs that have eluded production using established protocols despite aggressive efforts in many laboratories. In particular, several specificities to blood group antigens are needed to replace the deceasing supplies of human polyclonal antibodies including, for example: anti-Fy^a, anti-Fy^b, anti-S, anti-e, anti-Kp^a and anti-Js^a.

The experience of cloning the gene encoding the Duffy (gp-Fy) protein, of determining the molecular basis of the Fy^a/Fy^b polymorphism, of producing transgenic mice whose red blood cells (RBCs) express the human Fy^b antigen, have been found to make the Duffy polymorphism an ideal system in which to produce MAbs. Our development of MAbs anti-Fy^a and anti-Fy^b constitute a model system which demonstrates the general utility of using transgenic mice to develop MAbs for any polymorphism which is carried on a protein whose gene has been cloned and the molecular basis of which has been determined. Thus, a comprehensive range of MAbs can now be made as blood typing reagents, as tools for the investigation of biological function, and as possible therapeutic applications.

The polymorphic protein used in the method can be any protein having two or more immunologically differentiable forms. Generally, each form of the protein is encoded by an allele of the same gene. Thus, for any gene where two (or more) alleles are known and are

recognized as defining polymorphism in the expressed gene products, the method of the invention can be used to generate monoclonal antibodies against epitopes defined by the polymorphism, if such exist. The protein, therefore, is a protein which is at least potentially recognizable as non-self by an animal which does not express the particular protein.

The protein is preferably a protein which is expressed on or in a cell membrane, such as a cell surface antigen, e.g., a receptor, an enzyme, etc. One highly preferred protein is the Duffy antigen, also known as the gp-Fy protein, having a polymorphism characterized by gp-Fy^a and gp-Fy^b expressed proteins. The Duffy polymorphism is described in U.S. application Serial No. 08/140,797, filed on October 21, 1993, and in U.S. application Serial No. 08/749,543, filed on November 15, 1996, the entire disclosures of which are incorporated by reference herein.

Other blood group antigens can be used to develop monoclonal antibodies according to the method of the invention. For example, the Rh group and the Kell group are known to include allelic polymorphisms which can be exploited according to the invention. Other blood group proteins or red blood cell membrane proteins having known polymorphism are shown in Table 1.

TABLE 1

Some Red Cell Blood Group Antigens with One Amino Acid Substitution

Blood Group System	Antigen Polymorphism	Codon	Residue No.	Amino Acid
MNS	s⇒S	ATG⇒ACG	29	Met⇒Thr
	ENEH⇒Hut	ACG⇒AAG	28	Thr⇒Lys
	ENEH⇒VW	ACG⇒ATG	28	Thr⇒Met
Rh	C⇒c	TCT⇒CCT	103	Ser⇒Pro
	E⇒e	CCT⇒GCT	226	Pro⇒Ala
	Tar (-)⇒(+)	CTX⇒CCX	110	Leu⇒Pro
Kell	k⇒K	ACG⇒ATG	193	Thr⇒Met
	Kp^b⇒Kp^a	CGG⇒TGG	281	Arg⇒Trp
	Kp^b⇒Kp^c	CGG⇒CAG	281	Arg⇒Gln

	$Js^b \Rightarrow Js^a$	CTC⇒CCC	597	Leu⇒Pro
	K11⇒K17	GTC⇒GCC	302	Val⇒Ala
	$U1^a$(-)⇒(+)	GAT⇒GTA	494	Glu⇒Val
	K17(-)⇒(+)	GTC⇒GCC	302	Val⇒Ala
	K1 (-)⇒(+)	ACG⇒ATG	193	Thr⇒Met
Duffy	$Fy^b \Rightarrow Fy^a$	GAT⇒GGT	44	Asp⇒Gly
Diego	$Di^a \Rightarrow Di^b$	CCX⇒CTX	854	Pro⇒Leu
	$Wr^b \Rightarrow Wr^a$	GAG⇒AAG	658	Glu⇒Lys
	Wd(-)⇒(+)	GTG⇒ATG	557	Val⇒Met
	Rb^a (-)⇒(+)	CCA⇒CTA	548	Pro⇒Leu
	WARR(-)⇒(+)	ACX⇒ATX	552	Thr⇒Ile
	ELO(-)⇒(+)	CGG⇒TGG	432	Arg⇒Trp
	Fr^a(-)⇒(+)	GAG⇒AAG	480	Glu⇒Lys
	Wu(-)⇒(+)	GGC⇒GCC	565	Gly⇒Ala
	Bp ^{a}(-)⇒(+)	ACA⇒AAA	569	Asn⇒Lys
	Hg ^{a}(-)⇒(+)	CGT⇒TGT	656	Arg⇒Cys
	Mo ^{a}(-)⇒(+)	CGT⇒CAT	656	Arg⇒His
Yt	Yt $^a \Rightarrow Yt^b$	CAC⇒AAC	322	His⇒Asn
Colton	Co $^a \Rightarrow Co^b$	GCG⇒GTG	45	Ala⇒Val
Landsteiner-Wiener	LW $^a \Rightarrow LW^b$	CAG⇒CGG	70	Gln⇒Arg
Cromer	Cr^a (+)⇒(-)	GCA⇒CCA	193	Ala⇒Pro
	$Tc^a \Rightarrow Tc^b$	CGT⇒CTT	18	Arg⇒Leu
	$WES^b \Rightarrow WES^a$	CTX⇒CGX	48	Leu⇒Arg
Indian	$In^b \Rightarrow In^a$	CGG⇒CCG	26	Arg⇒Pro

The invention, therefore permits the generation of panels of blood typing antibodies (e.g., hemagglutination reagents) against any or all of the various expressed epitopes of any or all of these blood group proteins. But the invention is useful in general to develop monoclonal

antibodies against any polymorphic cell surface membrane protein, especially those proteins against which monoclonal antibody cannot be routinely made by conventional methods.

The invention can further be used to identify polymorphisms in proteins where none has been previously identified. For example, if two or more alleles of a gene are known, each encoding a different form of the protein in question, then the transgenic method can be used to determine whether such polymorphism corresponds to epitopic differences sufficient to induce an immune reaction. Similarly, other mechanisms associated with protein polymorphism, e.g., differential splicing at the mRNA level, can be studied and exploited by means of the invention. The induction of a cell secreting antibody specifically reactive with the protein used as the putative immunogen provides substantive evidence of epitopic difference. A utility of such an approach is found in the ability to generate information about a protein where conventional immunological characterization reagents and methods fail to yield adequate information. For example, conventional hydropathy analysis may produce ambiguity of interpretation as to whether a difference in primary structure lies within or without the cell membrane. The production of a monoclonal antibody having specificity for one form of the protein but not another would constitute evidence of a difference in extracellular conformation.

The cloning and sequencing of the human Duffy gene has enabled numerous utilities related to characterizing the normal physiological role of the gp-Fy protein as well as the abnormal role of the protein as a malarial binding ligand. Of particular interest for the present invention, the knowledge of the molecular basis of the Duffy blood group polymorphism enables the construction of transgenic animals to express heterologous Duffy protein. Various transgenic techniques are known, and certain of these techniques can yield heritability of the transgene. See, e.g., Pinkert et al. (1995) for an overview of these techniques, and the documents cited there for greater detail. For example, the invention takes advantage of transgenic mammals transformed by integration of an expressible transgene comprising a heterologous Duffy-related nucleic acid sequence into the genome of the mammal. Such transgenic animals express a Duffy protein normally expressed in erythrocytes of another species, preferably a human Duffy protein.

The animal used to create the transgenic model can be any species, but is preferably a mammal, for example, mice, rats, goats, sheep, pigs, cats, dogs, rabbits, horses or another

mammal. Mice are particularly preferred. Moreover, it is preferred that the animal to be transformed be inbred to have a high degree of genetic uniformity between individuals, so that no interfering immune response is generated upon introduction of the cells from one animal into another. Numerous strains of inbred animals, most notably mice, are commercially available. The skilled artisan will appreciate advantages derived from the animal having a relatively well characterized genome, and especially a well characterized immune system. It is further preferred that the autologous gene or genes, if any, which is(are) evolutionarily related to the heterologous gene be at least partially understood at the molecular level.

By employing transgenic animals expressing only one form of a polymorphic protein, the method of the invention ensures that the only immunological difference between cells of the transgenic mouse and the cells or protein used as the immunogen will be the polymorphism. This approach virtually guarantees that any immune response raised by the transgenic mouse will raise an immune response directed solely against the single polymorphic difference. This approach has the potential of allowing production of MAbs that are currently unavailable, as well as MAbs specific for antigens that require the milieu of the cell membrane to be expressed.

To date, no MAb anti-Fy^a or anti-Fy^b have been produced. Clinically significant antibodies of both specificities have been described (Giblett 1977; Badakere et al. 1970; Freisleben 1951; Beattie 1988) and it is important to be able to type donor and patient blood for the corresponding antigens in the pre-transfusion setting. Attempts to make specific MAb anti-Fy^a and anti-Fy^b have included injection of mice or rabbits with human RBCs, enriched Duffy protein or synthetic peptides, but these methods have been unsuccessful. Use of the purified Duffy protein or peptides as immunogens has resulted in production of antibodies to the Duffy protein but not to the polymorphism. These findings suggest that the Fy^a and Fy^b antigens are expressed only if the Duffy protein is within the milieu of the RBC membrane.

Thus, to resolve this ambiguity, the present method can be used to make Fy^a transgenic mice, with RBCs from these mice into bing injected into existing Fy^b transgenic mice. RBCs from the Fy^b transgenic mice are injected into the Fy^a transgenic mice. This cross-immunization protocol, i.e., immunization between allelic transgenic mice, provides an exquisite means by which to make MAbs with specificities not currently available. Indeed, following this approach, a more comprehensive range of MAb specificities can be made.

The Fy^a antigen was first discovered on erythrocytes in 1950 using an antibody (called anti-Fy^a) in the serum of a multitransfused hemophiliac patient (Cutbush 1950). Just a year later, its antithetical antibody, anti-Fy^b was reported (Ikin et al. 1951). The Duffy blood group system has expanded modestly over years, however, intense interest in Duffy was triggered when the gene encoding Duffy glycoprotein (gp-Fy) was cloned in 1993 (Chaudhuri et al. 1993), and the role of gp-Fy in the RBC membrane was elucidated (Neote et al. 1994; Horuk et al. 1993; Iwamoto et al. 1996).

Gp-Fy, which is predicted to have seven transmembrane α-helices, is encoded by a gene on the long arm of chromosome 1 (Donahue et al. 1968). The protein has an apparent molecular mass of M_r 35,000 to 43,000 on a polyacrylamide gel. The antigens Fy^a and Fy^b, located on the N-terminal extracelluar domain of gp-Fy (Fig. 1) (Chaudhuri et al. 1995; Iwamoto et al. 1995; Mallinson et al. 1995), define four phenotypes in this blood group system: Fy(a+b-), Fy(a-b+), Fy(a+b+) and Fy(a-b-). In whites, the first three phenotypes are commonly observed, and Fy(a-b-) individuals are extremely rare. However, the Fy(a-b-) phenotype among blacks is frequent in African-Americans and reaching almost 100% in people from some areas of West Africa (Race et al. 1975). In Fy(a-b-) individuals of African descent, the absence of Duffy antigens is associated with an absence of Duffy glycoprotein in the RBC membrane due to a single nucleotide substitution of GATA to GACA in the erythroid promoter (Tournamille et al. 1995). Thus, Duffy mRNA was detected in many other tissues in both Duffy positive and Duffy negative individuals (Neote et al. 1994; Iwamoto et al. 1996; Chaudhuri et al 1995).

Anti-Fy^a and anti-Fy^b are usually IgG molecules and are of variable clinical significance. Mild hemolytic transfusion reactions have been attributed to examples of these antibodies. Two other antibodies, anti-Fy3 and murine MAb anti-Fy6 bind to the Duffy glycoprotein (Albrey et al. 1971; Nichols et al. 1987). Fy3 and Fy6 are present on all RBCs except those of the Fy(a-b-) phenotype. Using a MAb anti-Fy3, the Fy3 antigen has been shown to reside on the last extracellular loop of the gp-Fy protein (Fig. 1) (Lu et al. 1995). A MAb anti-Fy6, produced by immunizing mice with pooled red cells (Riwom et al. 1994), binds to the N-terminal extracellular region between amino acids 31 and 40 of the gp-Fy protein (Hausman et al. 1996). The distribution of Fy6 on RBCs of non-human

primates differs from Fy^a, Fy^b and Fy3 (Nichols et al. 1987). MAb anti-Fy6 has been invaluable in the isolation of gp-Fy (Riwom et al. 1994; Chaudhuri et al. 1989).

Apart from the fact that Fy^a and Fy^b are immunogenic in humans and are frequently involved in blood transfusion, the biochemical and functional characterization of the gp-Fy is of major importance. In addition to cloning the gene encoding gp-Fy by Chaudhuri and coworkers (described in U.S. Application Serial No. 08/140,797, filed on October 21, 1993), the functions of gp-Fy as a chemokine receptor and its involvement in the process of invasion of human RBCs by malarial parasites *Plasmodium vivax* and *P. knowlesi* (a simian parasite) were elucidated (Chaudhuri et al. 1989; Miller et al. 1975; Miller et al. 1976; Barnwell et al. 1989). Horuk and coworkers (1993) showed that gp-Fy acts as a receptor for chemokines responsible for such processes as cell interaction, cell growth and inflammation, namely interleukin-8 (IL-8), melanoma growth stimulatory activity (MGSA), RANTES and monocyte chemotactic protein 1 (MCP-1). However, there is no subsequent signal transduction demonstrated upon binding. It has been suggested that gp-Fy absorbs excess cell-signaling molecules (Darbonne et al. 1991). Despite the intensive work performed on the gp-Fy, the role of Duffy antigens in invasion by malaria parasites and in the binding to chemokines is not clear. What is clear is that structural information on the gp-Fy is needed to understand the process of the interaction between the merozoite or chemical ligands and its RBC membrane receptor. Once anti-Fy^a and anti-Fy^b MAbs are available, they can be used to further investigate the specific topology of the gp-Fy protein that contributes to the Fy^a/Fy^b epitopes.

The genes encoding Fy^a and Fy^b antigens have been cloned. Transgenic mice have been constructed, whose RBCs express the human Fy(a-b+) phenotype, by injecting genomic DNA into mouse zygotes. This knowledge can be used to generate transgenic mice expressing the human Fy(a+b-) phenotype. The offspring of these transgenic mice are expected to carry either human Fy^a or Fy^b antigens on their RBCs. Blood cells isolated from one group of transgenic mice are used to immunize the other group. This approach overcomes the observed problem that certain antigens can only induce an immune response by their close relatives, but not by lower species. Furthermore, it significantly limits the contamination of antibodies obtained by injection of human RBCs into mice. Perhaps most significantly, the only difference between the RBCs of the transgenic mouse being immunized and the transgenic mice RBCs being used as the immunogen is the Duffy antigen. Thus, it is

highly probable that the mouse will mount an immune response to the Fy^a or Fy^b polymorphism. After immunization, the spleen of the immunized mice is isolated, fused to myeloma cells and processed by conventional hybridoma technique to select hybrids secreting anti-Fy^a and anti-Fy^b. Such MAbs will be useful in the replacement of human anti-serum reagents in the practice of blood typing and in the investigation of the topology and function of the Duffy glycoprotein.

In addition, study on the capability of stimulation of immune response with the truncated protein or the intact cells will provide important topological information of gp-Fy. Nonetheless, the transgenic mice constructed will be extremely useful to elucidate the functions and biological role of the Duffy protein, and that would serve as an excellent animal model to develop any possible therapeutic treatments for chemokine disorders or the infection of malarial parasite in the third world.

The following examples are provided to assist in a further understanding of the invention. The particular materials and conditions employed are intended to be further illustrative of the invention and are not limiting upon the reasonable scope thereof.

EXAMPLE 1: Preparation of Transgenic Mice Expressing gp-Fy^b Protein

Transgenic mice have been constructed to express the human Duffy gp-Fy^b antigen using a method substantially in accordance with that illustrated in Figure 2. A 3523 bp genomic DNA fragment containing *FY*B* coding sequence and ~1.5 kb upstream and ~1 kb downstream flanking sequences (SEQ ID NO:1; see also Figure 3A and 3B) was amplified by the polymerase chain reaction using *FY*-specific primers (sense: 5-CTGCAGGGGTAGATGCCCTTTCTC-3 (SEQ ID NO:2); antisense: 5-GAATTCCAAGCAGAAGATGAATC-3 (SEQ ID NO:3)). The amplified fragment was cloned in the pBluescript vector (Strategene). Plasmid DNA was purified by two-round centrifugation in CsCl gradients. The fragment containing the inserted genomic *FY*B* gene was excised by appropriate restriction enzymes and separated on a gel followed by DNA purification. The pure DNA fragment was reconstituted to a concentration of approximately 5 μg/mL and was used to construct transgenic mice.

The purified DNA fragment was micro-injected into the male pronucleus of fertilized eggs of the B6/CBA F1 mouse (Jackson Laboratory, Bar Harbor, ME), which had been

removed from the oviducts of a female mouse that had mated the night before. The zygotes with the insertion were transferred to the oviducts of 0.5-day pseudo-pregnant females and allowed to develop into embryos. Ten females became pregnant, producing 60 pups.

Four weeks after birth, DNA was prepared from tail clips of each baby animal using proteinase K digestion and ethanol precipitation. The DNA was tested for *FY* sequence integration by dot blot hybridization with a probe derived from the Duffy genomic DNA or by PCR amplification using *FY*-specific primers. In the PCR, 200 ng of the genomic DNA was amplified with the Duffy-specific primers with Taq polymerase. The PCR reaction was carried out for 30 cycles as follows: 30 s at 94°C, 30 s at 65°C, and 3 min at 72°C. Ten microliters (10 μL) of the reaction mixture was run on a 1% agarose gel, using a 1 kb DNA marker, with a non-transgenic mouse sample as a control. Figure 4 shows representative PCR results, with a DNA marker (lane 1), DNA from a non-transgenic mouse control (lane 2), and DNA samples from 12 transgenic mice (lanes 3-14). The dot blot hybridization and PCR amplification showed that 11 out of the 60 mice (18% transduction rate) had successful integration of the human Duffy genomic DNA into their chromosome, and more than one copy of *FY* was observed (data not shown).

Expression of the exogenous gene was examined by hemagglutination. Serological studies were performed by collecting blood from each animal showing successful integration of human Duffy DNA by puncture of the orbital plexus under an anesthetized condition. The isolated RBCs were tested for the presence of human Fy^b antigen by hemagglutination using murine MAbs anti-Fy3, anti-Fy6, and human anti-Fy^b. Of the 11 transgenic mice having the Duffy gene incorporated, RBCs from four mice showed the expression of the expected cognate antigens. These results are shown in Table 2, below, which summarizes immunological data concerning red cells from the 11 mice as compared to human red cells.

TABLE 2

Erythrocytes	anti-Fy3	Anti-Fy6	Anti-Fy^b	PCR
Human	++	++	++	Positive
Mouse #1	++++	++	++	Positive
Mouse #2	++++	++	++	Positive
Mouse #3	-	-	-	Positive
Mouse #4	-	-	-	Positive
Mouse #5	++	++	++	Positive
Mouse #6	-	-	-	Positive
Mouse #7	+++	+	++	Positive
Mouse #8	-	-	-	Positive
Mouse #9	-	-	-	Positive
Mouse #10	-	-	-	Positive
Mouse #11	-	-	-	Positive

These data indicate that not all of the integration of *FY*B* gene occurred at the chromosomal site which is being actively transcribed. However, approximately 7% of the transfected animals actively transcribed *FY* and synthesized (expressed) the Duffy Fy^b protein. Furthermore, it is demonstrated that the integrated DNA sequence contains all of the information necessary for Duffy promoter activity and its expression in erythroid specific manner. The red cells of the transgenic mice are serologically identical to a Duffy-positive human having Fy(a-b+) erythrocytes. These agglutination data imply that the expressed human Duffy protein was folded onto the mouse RBC membrane preserving its native (i.e., human) conformational structure and antigenic sites.

It is worth noting that random integration of the Duffy genomic DNA into mouse chromosome demonstrably works well in expression of the Duffy protein. Even so, targeted integration into an appropriate chromosome may be necessary for producing desired transgenic animals in other cases. Such targeted transformation can be accomplished by conventional methods, and given the information provided herein is within the skill of the artisan

EXAMPLE 2: Construction of Transgenic Mouse Expressing Human Fy^a Antigen

The method described in Example 1 is directly adaptable for the construction of a transgenic animal expressing the Fy^a antigen. In this case, the genomic DNA of *FY*A* are either amplified from an individual of Fy(a+b−) phenotype, as described above, or obtained by performing site-directed mutagenesis at nucleotide 131 of the open reading frame (ORF) (A^{131}→G). The Fy^a and Fy^b antigens differ as a result of single nucleotide difference (G^{131} or A) encoding amino acids Gly^{44} (Fy^a) or Asp (Fy^b) in the N-terminal extracellular domain of the Duffy glycoprotein. A vector is constructed containing the *FY*A* DNA, and mice are transfected. The resulting transformed mice express human Fy^a on their red cell surfaces, having a conformation suitable for producing an agglutination reaction identical to that of native human Fy^a. Once transformed, the transgenic mice can be bred to obtain the homozygous transgenic mice, and further bred to produce a stock of animals having the same DNA insertion.

EXAMPLE 3: Preparation of Soluble Peptides That Express Fy^a or Fy^b Antigens

To examine if the extracellular N-terminal 65 amino acids are sufficient to stimulate an immune response to Fy^a or Fy^b antigens, Duffy-based peptides can be synthesized using recombinant DNA technology. The fragment encoding the N-terminal hydrophilic domain of the gp-Fy is amplified by using appropriate primers (see Figures 3A and 3B) with a purification tag of six histidine and a proteolytic site in front of it. To ensure that the truncated gp-Fy is immunogenic, the recombinant peptide might require conjugation to an inert carrier (MAP core) or dimerized through a recombination PCR procedure. The resulting PCR fragment is sequenced to ensure the perfect amplification and then inserted into an eukaryotic expression vector. As Fy^b has been successfully expressed in K562 cells, a human erythroleukemic cell line (Chaudhuri et al. 1994), the eukaryotic vector can be transiently transfected into K562 cells by calcium phosphate technique. The supernatant containing the truncated version of gp-Fy is harvested between 48 to 72 hours after transfection and eluted through either an ion exchange column or an affinity column with the conjugation of murine MAb anti-Fy6. The purified protein can be injected into the transgenic mice as the appropriate immunogen.

EXAMPLE 4: Immunization of Fy^a and Fy^b Transgenic Mice Using Recombinant Peptides

Recombinant Fy^a peptide is injected into the Fy^b transgenic mice prepared in Example 1 using a standard immunization protocol (Rudbach et al. 1995). Likewise, recombinant Fy^b peptide is injected into the Fy^a transgenic mice prepared according to Example 2. Serum is collected and examined for the production of anti-Fy^a and anti-Fy^b, respectively. Since the human gp-Fy has become one of the native proteins on the RBCs of these transgenic mice during embryonic development, immunization with human-origin antigen will likely produce a stronger immune response in them as compared to that in the original nontransgenic mice. The immunogenicity of the recombinant peptide is optimized through the use of appropriate adjuvants to yield highly quantitative and qualitative antibodies.

If specific antibody is produced in response to the injected peptide, the monoclonal hybridoma technique is employed as described below. Otherwise, if the soluble peptide does not induce an immune response, this may indicate that the epitopes of Fy^a and Fy^b are associated with other regions (e.g., the extracellular loops) of the Duffy protein or with other membrane proteins. This would suggest that the entire Duffy molecule with a mature conformation on the RBC membrane is needed to create the proper epitope for immunization. Thus, the RBCs of the transgenic mice would be the best immunogen. Moreover, in order to evaluate whether the transgenic mouse that expresses the native human Duffy protein is indeed a better host for immunization, the same procedure can be performed using an original untreated strain.

EXAMPLE 5: Immunization Using Transgenic Mice RBCs Expressing Fy^a or Fy^b

Once the transgenic strains of Fy^a and Fy^b mice are established, a cross-immunization procedure is performed generally in conformity with the method illustrated in Figure 5. The RBCs isolated from each mouse strain are injected subcutaneously and intravenously into the mice of their counter group. We have shown that Fy^b antigens are expressed on the transgenic mouse RBCs as indicated by specific human antiserum (Table 2). As these two sources of RBCs only differ in the expression of either Fy^a antigen or Fy^b antigen, and not any other antigenic sites of the Duffy protein (e.g., Fy3 and Fy6) or other RBC-borne antigens, the polymorphic sites of Fy^a or Fy^b are the only target to produce antibody. This results in a

greater chance of making anti-Fy^a and anti-Fy^b since the presumably higher immunogenic epitopes to mouse immune system are unrecognizable in this protocol. This assumption is based on the finding that immunization of mice with RBCs, purified gp-Fy, or synthetic peptides has only resulted in production of antibodies to the gp-Fy and not to the Fy^a/Fy^b polymorphism.

EXAMPLE 6: Immunization of Non-Transgenic Mice with RBCs from a Transgenic Fy^b Mouse

Since transgenic mice whose RBCs express human Fy^b antigens are available, the cells of these mice can be used to immunize mice of the same wild type strain. If the non-transgenic mice mount an immune response, the preparation of MAbs can be usefully attempted.

EXAMPLE 7: Fusion and Cloning of the Hybridoma Cells Secreting Anti-Fy^a/Fy^b

After final immunization, the mice selected according to satisfactory antibody titers are killed and their spleens are removed. The splenocytes are fused into the mouse myeloma X63-Ag8.653 cells using a standard polyethylene glycol (PEG)/dimethylsulfoxide (DMSO) procedure, followed by HAT selection (Rudbach et al. 1995; Gorny et al. 1994). The mouse myeloma used for fusion is usually a HAT-sensitive variant of the Balb/c-derived myeloma. Although the spleen cell donor is an inbred strain other than Balb/c, the MAb is generated only from cell culture fluids, thereby avoiding the histocompatibility problem. Alternatively, the F1 progeny of a Balb/c:"spleen cell donor" cross, which contains both sets of histocompatibility antigens, can be used to grow the hybridomas.

The supernatant of the hybridoma cells is screened for antibody secretion with antigen-positive and antigen-negative human RBCs by direct hemagglutination or ELISA. Hybridoma cells producing specific antibody are cloned by limiting dilution. The stable cell lines are frozen for future use or expanded in culture for bulk production of antibodies. By this protocol, both IgG and IgM antibodies are obtained.

This result makes possible the use of direct agglutinating monoclonal anti-Fy^a and anti-Fy^b for screening programs to find antigen-negative donors, since currently available reagents require the antiglobulin test and cannot be used to type RBCs with a direct antiglobulin test.

EXAMPLE 8: Evaluation of the Monoclonal Anti-Fy^a and Anti-Fy^b

The monoclonal anti-Fy^a and anti-Fy^b are evaluated for their specificity and sensitivity in comparison to the current reagents by using a large number of blood samples from random donors and by testing rare RBCs with known phenotypes, which are commercially available. Standard hemagglutination techniques are used.

EXAMPLE 9: Characterization of the Monoclonal Anti-Fy^a and Anti-Fy^b Antibodies

The nucleotide sequences of variable domains of anti-Fy^a and anti-Fy^b are identified by sequence analysis with specific primers for this region. As the Fy^a/Fy^b antigen is suggested to be involved in the chemokine binding and in the parasite invasion, the obtained information will be extremely useful for design of an immunotherapeutic agent or a "vaccine" for blocking the interactions between the Duffy protein and its ligands or the parasites.

Thus, while there have been described what are presently believed to be the preferred embodiments of the present invention, those skilled in the art will realize that other and further embodiments can be made without departing from the spirit of the invention, and it is intended to include all such further modifications and changes as come within the true scope of the claims set forth herein.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: METHOD OF MAKING MONOCLONAL ANTIBODIES USING POLYMORPHIC TRANSGENIC ANIMALS

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3523 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATTCCAAG CAGAAGATGA ATC 23

WHAT IS CLAIMED IS:

1. A method for making an antibody, comprising:

constructing a first transgenic animal to express a first form of an exogenous polymorphic protein encoded by a first allele of a gene encoding the protein;

constructing a second transgenic animal to express a second form of the polymorphic protein encoded by a second allele of the gene encoding the protein;

immunizing said first transgenic animal with cells from said second transgenic animal expressing said second form of the polymorphic protein to induce an immune response in said first transgenic animal yielding an antibody specific for an epitope characteristic of said second form of the polymorphic protein; and

isolating the antibody.

2. A method according to Claim 1, further comprising isolating from said first transgenic animal a lymphoid cell capable of producing said antibody.

3. A method according to Claim 2, further comprising fusing said antibody-producing lymphoid cell with an immortal cell to provide an antibody-producing hybridoma cell.

4. A method according to Claim 1, wherein said polymorphic protein is expressed on a cell membrane.

5. A method according to Claim 4, wherein said polymorphic protein is a blood group protein.

6. A method according to Claim 5, wherein said blood group protein is gp-Fy protein.

7. A method according to Claim 1, wherein said polymorphic protein is a human polymorphic protein.

8. A method according to Claim 1, wherein said first transgenic animal and said second transgenic animal are both transformed from animals of one inbred strain.

9. A method according to Claim 1, wherein said first and second transgenic animals are mice.

10. A hybridoma cell that cannot be routinely made by standard methods, produced by a method comprising:

constructing a first transgenic animal to express a first form of an exogenous polymorphic protein encoded by a first allele of a gene encoding the protein;

constructing a second transgenic animal to express a second form of the polymorphic protein encoded by a second allele of the gene encoding the protein;

immunizing said first transgenic animal with cells from said second transgenic animal expressing said second form of the polymorphic protein to induce an immune response in said first transgenic animal yielding an antibody specific for an epitope characteristic of said second form of the polymorphic protein;

isolating from said first transgenic animal a lymphoid cell capable of producing said antibody; and

fusing said antibody-producing lymphoid cell with an immortal cell to provide an antibody-producing hybridoma cell.

11. A hybridoma cell according to Claim 10, wherein said polymorphic protein is a blood group protein, and said hybridoma cell is capable of producing an antibody specific for an epitope characteristic of said second form of said blood group protein.

12. A hybridoma cell according to Claim 11, wherein said blood group protein is gp-Fy protein, and said hybridoma cell is capable of producing an antibody specific for an epitope characteristic said second form of gp-Fy protein.

13. An antibody specific for an epitope characteristic of one form of a polymorphic protein, produced by a method comprising:

constructing a first transgenic animal to express a first form of an exogenous polymorphic protein encoded by a first allele of a gene encoding the protein;

constructing a second transgenic animal to express a second form of the polymorphic protein encoded by a second allele of the gene encoding the protein;

immunizing said first transgenic animal with cells from said second transgenic animal expressing said second form of the polymorphic protein to induce an immune response in said first transgenic animal yielding an antibody specific for an epitope peculiar to said second form of the polymorphic protein; and

isolating said antibody.

14. An antibody according to Claim 13, wherein said method further comprises:

isolating from said first transgenic animal a lymphoid cell capable of producing said antibody; and

fusing said antibody-producing lymphoid cell with an immortal cell to provide a hybridoma cell which produces said antibody.

15. A monoclonal antibody according to Claim 13, wherein said polymorphic protein is a blood group protein, and said monoclonal antibody is specific for an epitope characteristic of said second form of said blood group protein.

16. A monoclonal antibody according to Claim 13, wherein said blood group protein is gp-Fy protein and said monoclonal antibody is specific for an epitope characteristic of said second form of gp-Fy protein.

17. A method for making an antibody, comprising:

constructing a transgenic animal to express a first form of an exogenous polymorphic protein encoded by a first allele of a gene encoding the protein;

immunizing said transgenic animal with a peptide comprising an epitope characteristic of a second form of the polymorphic protein to induce an immune response in said transgenic animal yielding an antibody specific for said epitope.

18. A method according to Claim 17, further comprising isolating from said transgenic animal a lymphoid cell capable of producing said antibody.

19. A method according to Claim 18, further comprising fusing said antibody-producing lymphoid cell with an immortal cell to provide an antibody-producing hybridoma cell.

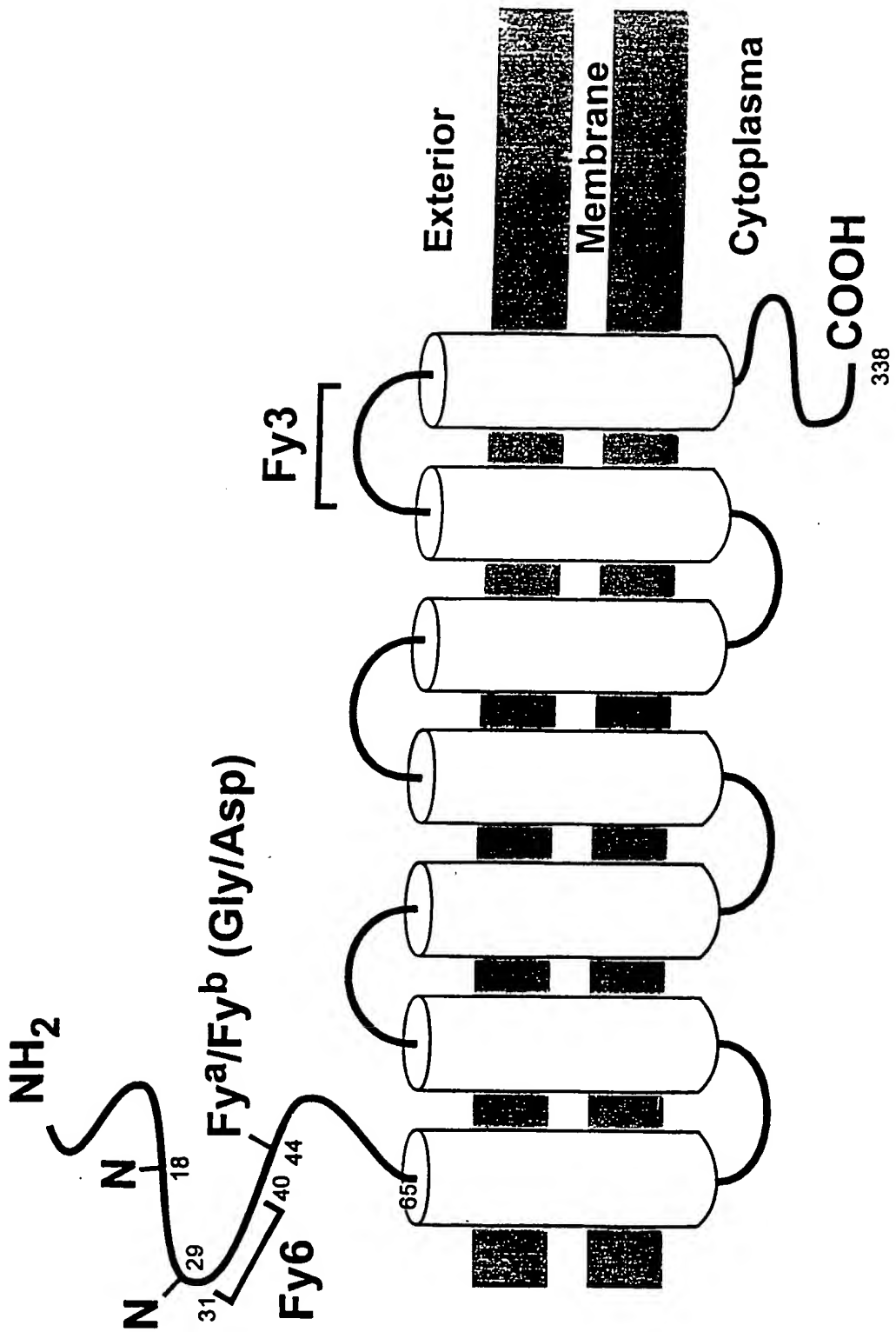
20. A method according to Claim 17, wherein said polymorphic protein is expressed on a cell membrane.

21. A method according to Claim 20, wherein said polymorphic protein is a blood group protein.

22. A method according to Claim 21, wherein said blood group protein is gp-Fy protein.

23. A method according to Claim 17, wherein said polymorphic protein is a human polymorphic protein.

24. A method according to Claim 17, wherein said transgenic animal is a mouse.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N
18
N
29
31
Fya/Fyb (Gly/Asp)
40
44
Fy6
65
Fy3
Exterior
Membrane
Cytoplasma
COOH
338

FIGURE 1

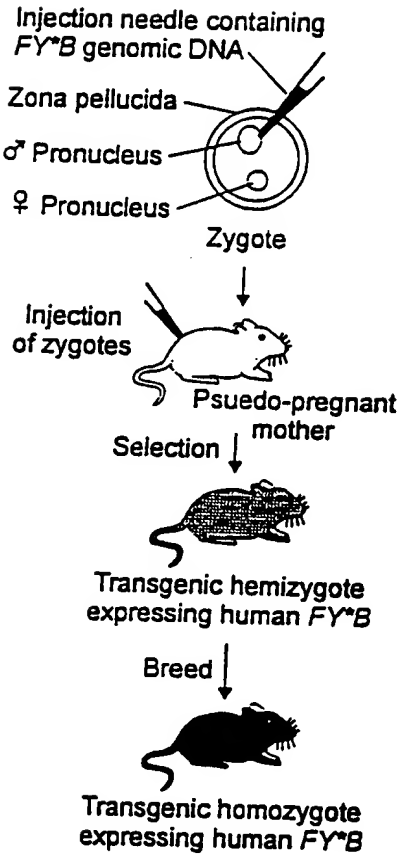
Injection needle containing
FY*B genomic DNA
Zona pellucida
♂ Pronucleus
♀ Pronucleus
Zygote
Injection
of zygotes
Psuedo-pregnant
mother
Selection
Transgenic hemizygote
expressing human FY*B
Breed
Transgenic homozygote
expressing human FY*B

FIGURE 2

Sense-primer for DNA amplification

```
CTGCAGGGGT AGATGCCCTT TCTCTCTGCT GGCCAGCTCT GCCCCTCAGT GAGAAACTTT    60
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AATATGAATA GAAATCACCC TGTGGGCAAT GGTCCCATTT TAAAATATGC TGTCCCATTG   180
TCCCCTAGAG CCTACTTTAA CTTGTCAGAC CATGTATTCC ACTTCATATG CAAGAGGCAT   240
GCACTGAGCC CATAGGTGGC TAGGCAAACA CCCAATAGCT CCCTGAAATG GCTTCATTAT   300
GGAGGCTCGA CAGCCACCCC AACCCTCCCA CTCTCACACT GAAACACCCA GACCTAGAGA   360
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CACAGCAAAT GTACACAGAG TTCAGTACAC ACAAAGAGCT CACGCCCACG TGCACACACC   480
CCTCAGTTGG GACAGAGTTG ACCACCACCA CCTTTCTCCC AAACACATGG CTTTGGAACT   540
GCCTTTCCTT GGATCCAGTT CAAGGGGATG GAGGAGCAGT GAGAGTCAGC CGCCCTTCCA   600
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CCTTCCTTGT GCTTGAAGAA TCTCTCCTTG CTGGAAAGCC CCCTGTTTTC TCAATCTCCC   720
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Initial codon of Duffy GP open reading frame

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TCCTATGGTG TGAATGATTC CTTCCCAGAT GGAGACTATG ATGCCAACCT GGAAGCAGCT  1680
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G in *FY*A* Primer for mutagenesis

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GCCCCCTGCC ACTCCTGTAA CCTGCTGGAT GACTCTGCAC TGCCCTTCTT CATCCTCACC  1740
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FIGURE 3A

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CTCACTGTGG GAATTTGGGG AGTGGCTGCC CTACTGACAC TGCCTGTCAC CCTGGCCAGT  2100
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CACCAGGCCA CCCGCACCCT CTTGCCCTCT CTGCCCCTCC CTGAAGGATG GTCTTCTCAT  2520
CTGGACACCC TTGGAAGCAA ATCCTAGTTC TCTTCCCACC TGTCAACCTG AATTAAAGTC  2580
                    *Stop codon
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GCATGACCTG AATGTGTGTG TGTGCGTGTG TGTGTGTGTG TGTACACATC TGTTTCTCGA  3180
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GGACCCAAAC CCCCACTCAA TTCTGCACTC ATCCCTGTTC ACAGGATATA GAATCGGGAT  3360
TTATGACTCA CTCCTTACCC AAATGAGTTT TCTTTACCCT GGTTTTTAAG CCTAGTCTTT  3420
TCTGTGTAGG ATGTGTGGAG GGAAGAAAAG ATCAAGAAGT TGTGAGGGGT GGAGAAACTT  3480
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                      Anti-sense primer for DNA amplification
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FIGURE 3B

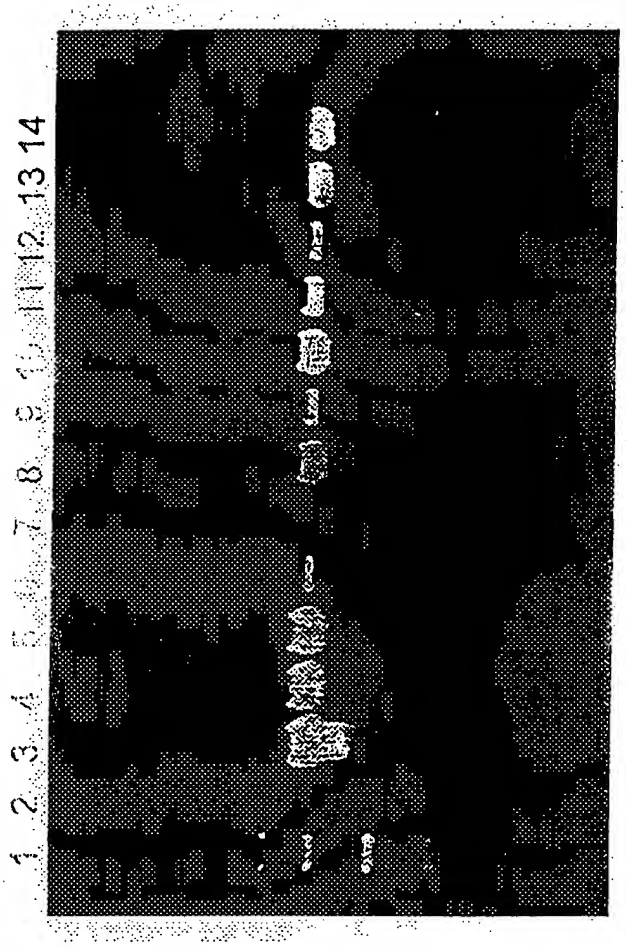


Fig. 4

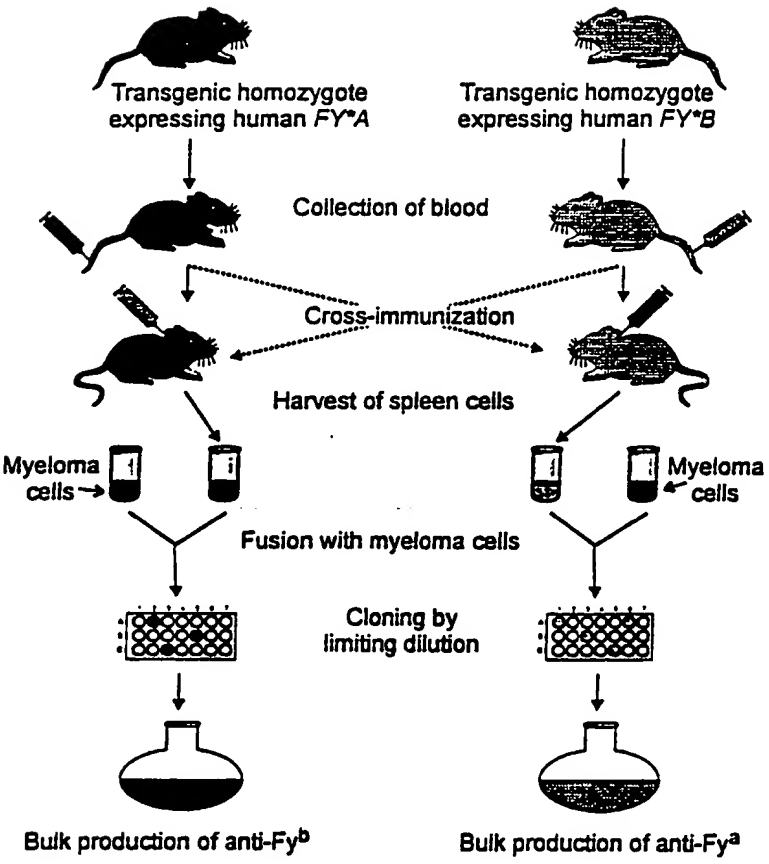

Transgenic homozygote expressing human FY*A
Transgenic homozygote expressing human FY*B
Collection of blood
Cross-immunization
Harvest of spleen cells
Myeloma cells
Myeloma cells
Fusion with myeloma cells
Cloning by limiting dilution
Bulk production of anti-Fyb
Bulk production of anti-Fya

FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/20783

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 5/28, 5/24, 15/00, 15/09, 15/63, 5/10
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 430/320.1, 6, 69.1, 375; 800/2; 935/89, 62, 70, 93; 536/23.5; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSIS, MEDLINE, EMBASE
search terms: transgenic, immunize, antibodies, hybridoma

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	HULTGREN et al. Antibodies to the hepatitis B e antigen (HBe-Ag) can be Induced in HBe-Ag-transgenic mice by adoptive transfer of a specific T-helper 2 cell clone. Clinical and Diagnostic Laboratory Immunology. September 1997, Vol. 4, No. 5, pages 630-632, see entire document.	1-24
Y	JAKOBOVITS et al. Production of Fully Human Antibodies by Transgenic Mice. Current Opinion in Biotechnology. October 1995, Vol. 6, No. 5, pages 561-566, see entire document.	1-24
Y	US 5,101,017 (RUBINSTEIN et al.) 31 March 1992, entire document, especially claims.	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
04 FEBRUARY 1998	23 FEB 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KAREN M. HAUDA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/20783

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

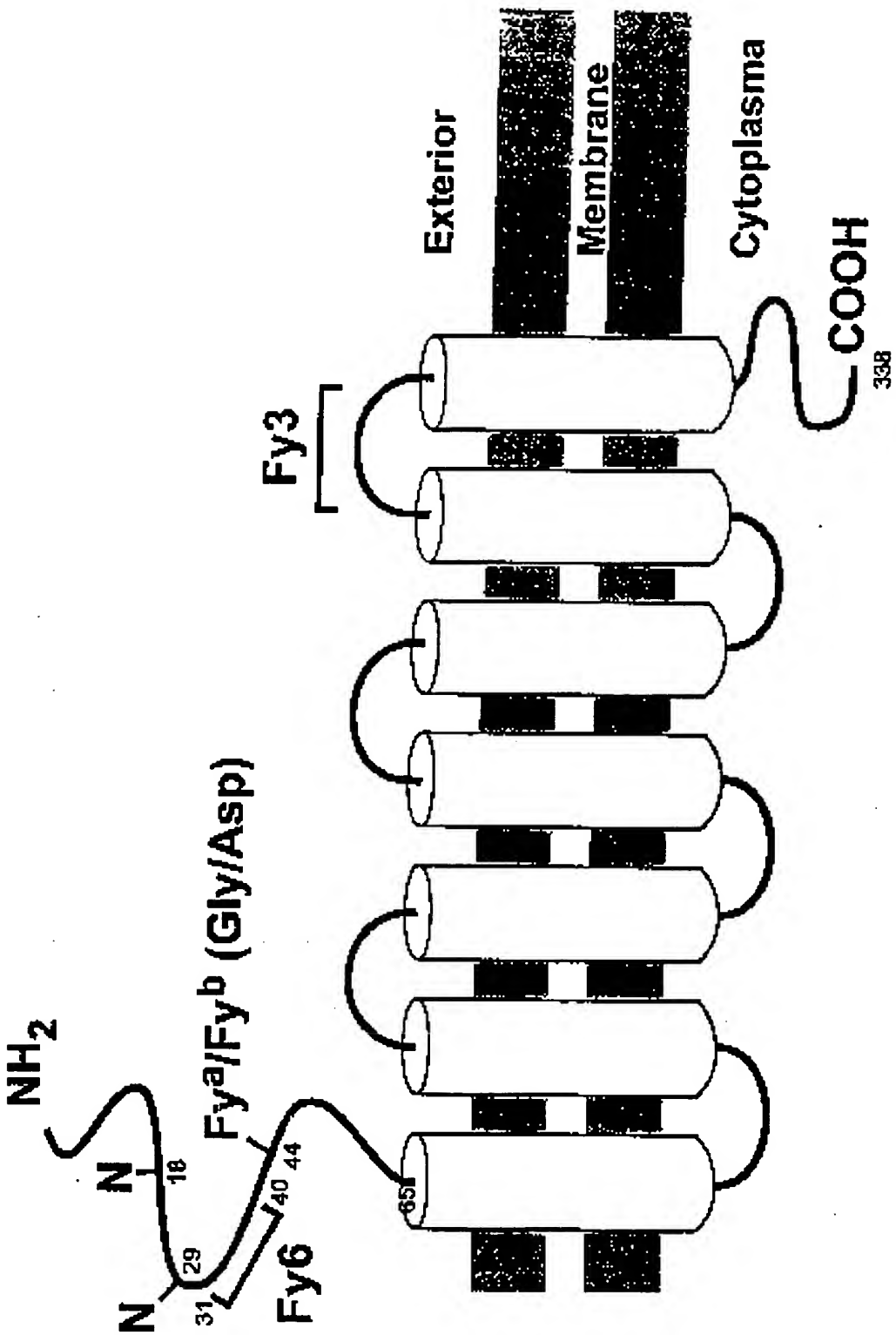
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHAUDHURI et al. Purification and Characterization of an Erythrocyte Membrane Protein Complex Carrying Duffy Blood Group Antigenicity. Journal of Biological Chemistry. 15 August 1989, Vol. 264, No. 23, pages 13770-13774, see entire document.	1-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/20783

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

430/320.1, 6, 69.1, 375; 800/2; 935/89, 62, 70, 93; 536/23.5; 514/2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Fy6

FIGURE 1

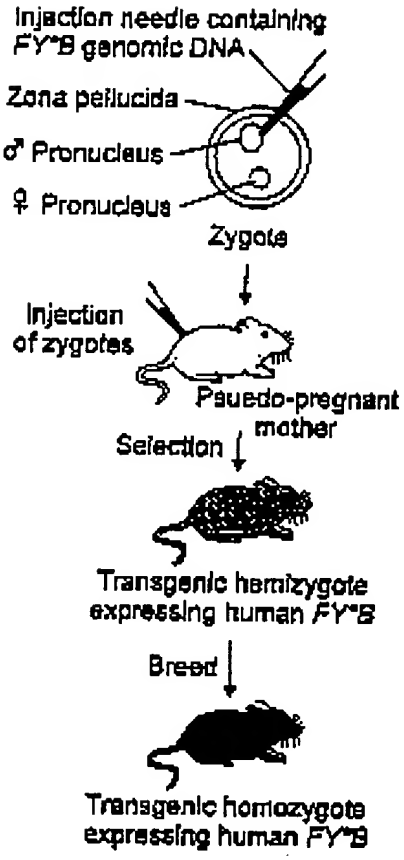
Injection needle containing FY*B genomic DNA
Zona pellucida
♂ Pronucleus
♀ Pronucleus
Zygote
Injection of zygotes
Pseudo-pregnant mother
Selection
Transgenic hemizygote expressing human FY*B
Breed
Transgenic homozygote expressing human FY*B

FIGURE 2

Sense-primer for DNA amplification

CTGCAGGGG? AGATGCCCTT TC?CTCTGCT GGCCAGCTCT GCCCCTCAG? GAGAAACTT? 60

ACATA?TGCT AAGATGCCTG GCCAATGAAA CAGT?CCAGA GAC???TATGT CCCCAGTAGA 120

AATA?GAATA GAAATCACCC TGT6GGCAA? GGTCCCATTT TAAAATATGC TGTCCCATTG 180

?CCCCTAGAG CCTACTTTAA C?TGTCAGAC CATGTAT?CC ACTTCATATG CAAGAGGCAT 240

GCAC?GAGCC CATAGGTGGC TAGGCAAACA CCCAATAGCT CCCTGAAATG GCTTCATTAT 300

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TAGCTAGACA CACCCAGACA CCCGCCAAGC CCC?CACATA CAGATATGTG CACAATGATA 420

CACAGCAAAT GTACACAGAG TTCAGTACAC ACAAAGAGCT CACGCCCACG TGCACACACC 480

CCTCAGTTGG GACAGAGT?G ACCACCACCA CCT??CTCCC AAACACATGG CTTTGGAACT 540

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CC??CCTTGT GCTTGAAGAA ?CTCTCCTTG CTGGAAAGCC CCCTGTTTTC ?CAATCTCCC 720

TTTCCACTTC GGTAAAATCT CTCCTTGC?G GAAAGCCCCC ?GTTTTCTCA ATCTCCCTTT 780

CCACT?CGGT AAAATGCCCA CTTTCTGGTC CCCACCTTTT TCCTGAGTGT AGTCCCAACC 840

AGCCAAATCC AACCTCAAAA CAGGAAGACC CAAGGCCAGT GACCCCCATA GGCCTGAGGC 900

TTGTTGCAGG CAGTGGGCGT GGGGTAAGGC TTCCTGATGC CCCCTGTCCC TGCCCAGAAC 960

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CAGCCCTTCT GTCTGCGGGC CTGAACCAAA CGGTGCCATG GGGAACTGTC TGCACAGGGT 1080

GAGTATGGGG CCAGGCCCCA GAGTCCCTTA TCCCTATGCC CCTCATTTCC CCTGCTGTTT 1140

GCCCCTCAGT CTTTATATCT CTTCCTTTTC CTCCTCATCT TTTCTCCCTT CCTGCTTTTT 1200

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TGACCTTGCA CTGCTCCTCC AGCCCCAGCT GCCCTGGCTT CCCCAGGACT GTTCCTGCTC 1380

CGGCTCTTCA GGCTCCCTGC TTTGTCCTTT TCCACTGTCC GCACTGCATC TGACTCCTGC 1440

AGAGACCTTG TTCTCCCACC CGACCTTCCT CTCTGTCCTC CCCTCCCACC TGCCCCTCAG 1500

Initial codon of Duffy GP open reading frame

TTCCCAGGAG ACTCTTCCGG TGTAACTCTG ATGGCCTCCT CTGGGTATGT CCTCCAGGCG 1560

GAGCTCTCCC CCTCAACTGA GAACTCAAGT CAGCTGGACT TCGAAGATGT ATGGAATTCT 1620

TCCTATGGTG TGAATGATTC CTTCCCAGAT GGAGACTATG ATGCCAACCT GGAAGCAGCT 1680

G in FY*A Primer for mutagenesis

GCCCCCTGCC ACTCCTGTAA CCTGCTGGAT GACTCTGCAC TGCCCTTCTT CATCCTCACC 1740

FIGURE 3A

```
AGTGTCCTGG GTATCCTAGC TAGCAGCACT GTCCTCTTCA TGCTTTTCAG ACCTCTCTTC  1800
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GGTGCTTCTG GTGGACTCTG CACCCTGATA TACAGCACGG AGCTGAAGGC TTTGCAGGCC  2160
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GCAGAAGCCC TGGCAATTTT GCACTGTGTG GCTACGCCCC TGCTCCTCGC CCTATTCTGC  2460
CACCAGGCCA CCCGCACCCT CTTGCCCTCT CTGCCCCTCC CTGAAGGATG GTCTTCTCAT  2520
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                     * Stop codon
TACACTGCCT TTGTGAAGCG GGTGGTTTCT TATTTTGTCT GGGGAGAAGA AGGAGAATGG  2640
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CCTCCCCCTG TCCAACCTGC CCAGCCTCTG TCTTCTGTCT CCTCTCTGGC TTTGTTTAGT  3000
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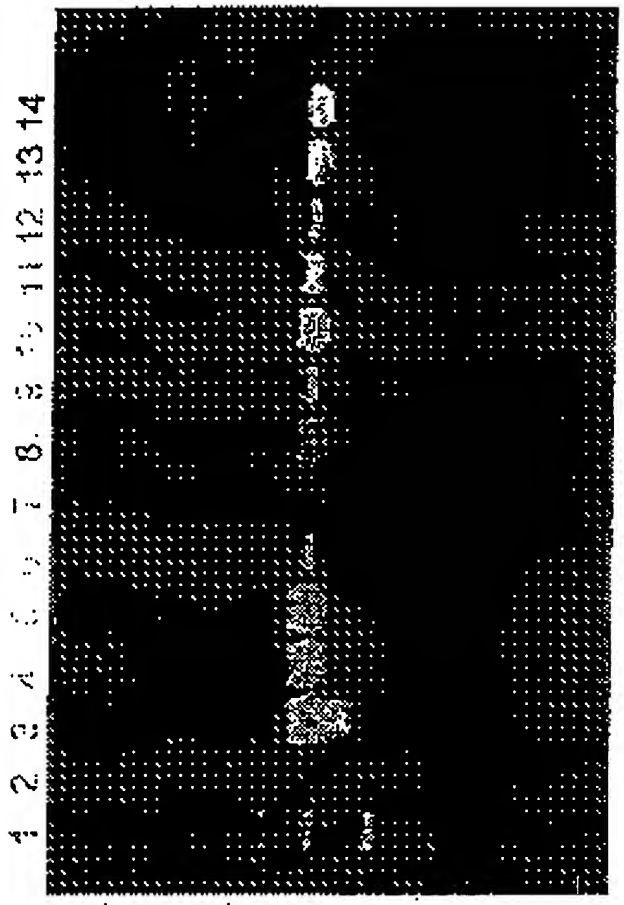
FIGURE 3B

Fig. 2



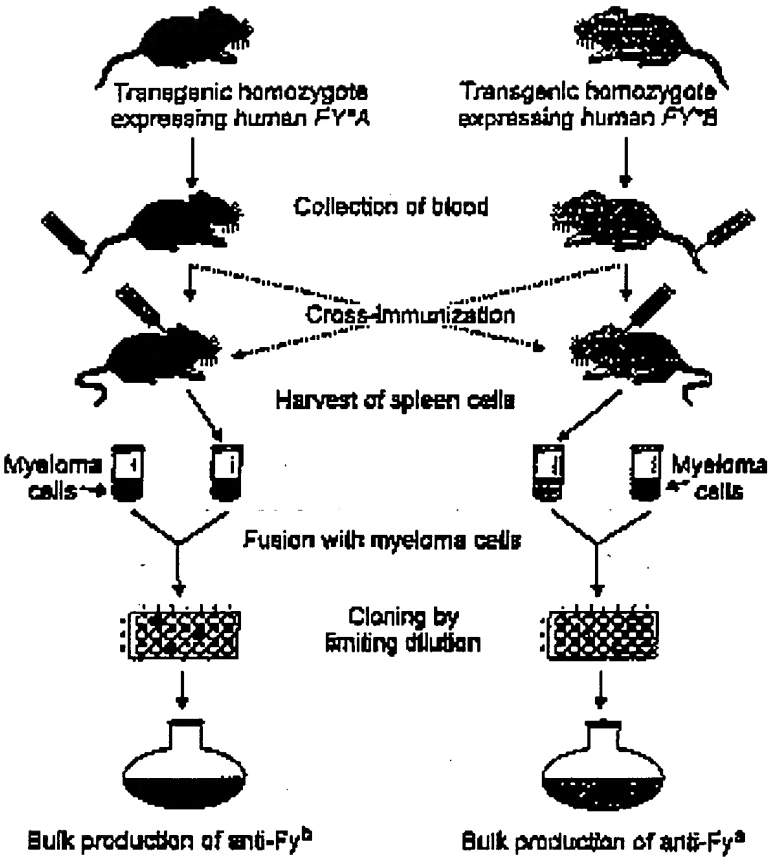

Transgenic homozygote expressing human FY*A
Transgenic homozygote expressing human FY*B
Collection of blood
Cross-immunization
Harvest of spleen cells
Myeloma cells
Myeloma cells
Fusion with myeloma cells
Cloning by limiting dilution
Bulk production of anti-Fy^b
Bulk production of anti-Fy^a

FIGURE 5